Immunologic Characterization of HIV-Specific DNA Vaccine

Norihisa Ishii, Yasuyuki Sugita,* Li-Juan Liu,† Setsuko Watabe,† Sumako Toda,† Ke-Qin Xin,† and Kenji Okuda†

Leprosy Research Center, National Institute of Infectious Diseases, Higashi-murayama, Tokyo; Department of *Dermatology and †Bacteriology, Yokohama City University School of Medicine, Yokohama, Japan

We developed a method for applying HIV-1 DNA vaccine topically in mice. Topical application of DNA vaccine to the skin is useful against infections. To find a less expensive and less cumbersome vaccination method, we administered HIV-1 DNA vaccine to the skin of mice after elimination of keratinocytes using a fast-acting adhesive. HIV-1 DNA vaccine induced high levels of both humoral and cell-mediated immune activity against HIV-1 envelope antigen. A high level of HIV-1-specific cytotoxic T lymphocyte response was also observed, and a high level of IFN-γ and IL-4 production was induced by the improved skin application of DNA vaccine. High levels of both HIV-specific cytotoxic T lymphocyte and delayed type hypersensitivity in topical application were induced by coadministration of the DNA vaccine with IL-12 expression plasmids and granulocyte-macrophage colony-stimulating factor expression plasmids. These immune responses were inhibited by intradermal injection of anti-CD11c or anti-I-A/I-E antibody. Therefore, topical administration of DNA vaccine is an effective route, and may be very useful for the prevention of infectious diseases. ***Key words: adjuvant/topical application/vaccination. Journal of Investigative Dermatology Symposium Proceedings 6:76–80, 2001***

The induction of a strong and long-lasting immunity is one of the most important elements in developing an effective human immunodeficiency virus (HIV) vaccine. Vaccines have been used to overcome many dangerous infections. Standard vaccines, such as live vaccines, attenuated live vaccines, and killed vaccines, vary in the kind and duration of security they provide. DNA vaccines have the advantage of inducing the production of protein antigens from DNA in host cells that exhibit natural glycosylation and processing, as is seen with the use of live attenuated vaccines (Hassett and Whitton, 1996; Donnelly *et al*, 1997; Ishii *et al*, 1997a; Weiner and Kennedy, 1999).

We have been developing an HIV-1-specific DNA vaccine candidate that is constructed from HIV-1 *env* and *rev* genes. DNA vaccine for HIV-1 has now been proved to induce high levels of both humoral and cell-mediated immune responses. We have previously reported (Okada *et al*, 1997; Toda *et al*, 1997; Tsuji *et al*, 1997a, b; Sasaki *et al*, 1998) that DNA vaccine induces strong cell-mediated immune responses, namely, cytotoxic T lymphocyte (CTL) response, delayed type hypersensitivity (DTH) response, and T cell proliferation response. We have recently demonstrated how the immunogenicity of DNA vaccine varies depending on the route of immunization in mice (Okada *et al*, 1997; Toda *et al*, 1997; Tsuji *et al*, 1997a, b; Sasaki *et al*, 1998; Xin *et al*, 1998; Liu *et al*, in press). The vaccines usually are derived by injection, commonly into muscle, which puts genes directly into cells and also leads to uptake by cells in the vicinity of the inserted needle. But in developing countries, simpler devices than needles are necessary.

In this review, we demonstrate the immunologic and characteristic importance of DNA vaccine. The mechanism of improved topical application of DNA vaccine to induce immune response is also discussed.

CHARACTERISTICS OF DNA VACCINE AND CYTOKINE EXPRESSION PLASMID

The immunogenic DNA plasmids, pCMV160IIIB which encodes glycoprotein 160 (gp160) of HIV-1 IIIB and pcREV which encodes HIV-1 REV (hereafter referred to as IIIB/REV), were described in our previous report (Okuda *et al*, 1995). Although our DNA vaccine formulation was designed to elicit an *env*-specific immune response, the *rev* expression plasmids were included because the expression of *env* protein is dependent on *rev* coexpression (Okuda *et al*, 1995) (**Fig 1**). Mouse IL-12 expression plasmid, designated pCAGGS IL-12, was previously described (Okada *et al*, 1997). Both mouse IL-12 p35 and p40 cDNA were inserted into the *Eco*RI site of the same pCAGGS expression vector (pIL-12). The mouse GM-CSF expression plasmid, designated pCAGGS GM-CSF (pGM-CSF), was provided by Dr. M. L. Disis, University of Washington, Seattle, WA (Disis *et al*, 1996).

IMMUNOGENICITY OF VARIOUS ROUTES OF IMMUNIZATION

We applied three different immunization methods, intramuscular (i.m.), intranasal (i.n.), and topical application. The detailed methods of i.m. (Okuda *et al*, 1995) and i.n. (Okada *et al*, 1997) immunization were described previously. Topical appli-

Manuscript received June 14, 2001; accepted for publication June 14, 2001.

Reprint requests to: Dr. Norihisa Ishii, Leprosy Research Center, National Institute of Infectious Diseases (NIID), 4-2-1 Aobacho, Higashi-murayama, Tokyo 189-0002, Japan. Email: norishii@nih.go.jp

Abbreviations: CTL, cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIV, human immunodeficiency virus; i.m., intramuscular; i.n., intranasal.

1087-0024/01/$15.00 · Copyright © 2001 by The Society for Investigative Dermatology, Inc.

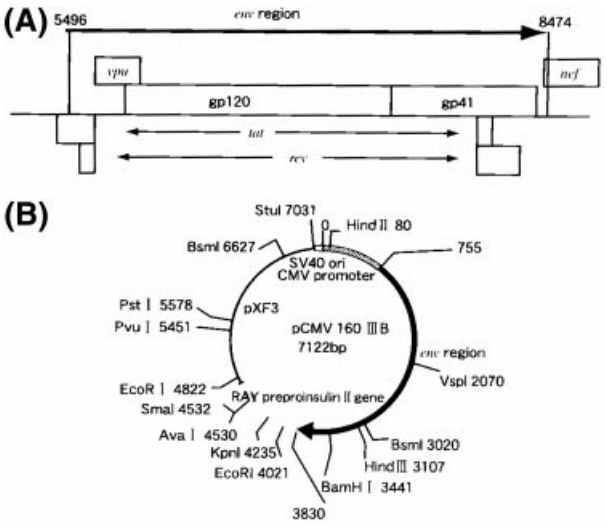

Figure 1. A gp160-expressing plasmid driving the CMV promoter. (*A*) Gene organization in the env region fragment (base position 5496–8474; Okuda *et al*, 1995) inserted into the expression plasmid. (*B*) Plasmid construction.

cation was conducted as follows. All hairs of an area on the back of a mouse were pulled out and the area was disinfected with 70% ethanol. Next, strong fast-acting adhesive (Alon Alfa, TOA, Tokyo, Japan) was smeared on a glass slide to cover approximately 0.5 cm^2 and stuck to the back of the mouse near the rump. After an interval of 20–30 s, the slide was ripped off so that the keratinous layer of the skin was removed with it. A solution of DNA-expressing plasmids dissolved in 20–30 μl of saline was applied to the newly exposed skin region to achieve immunization. This procedure was performed on days 0, 7, and 14 of the experiment (Liu *et al* in press). The immune response induced by DNA vaccine differed according to the route of immunization (i.m., i.n., or topical). As shown in **Fig 2**, topical application without stripping the skin induced weak antibody response or weak DTH response, even though 100 μg of DNA vaccine was applied (Liu *et al*, in press); however, topical application of DNA vaccine to the skin with stripping was a useful route of immunization against HIV. It induced strong HIV-1-specific immune response, and remained detectable after 3 mo in all immunization methods. We demonstrated that strong fast-acting adhesive adhesive (Alon Alfa) successfully removed the keratinocytic layers to uncover potently antigenic dendritic cells near the skin surface. Although it would not be the best way to obtain the same condition in clinical use, it is of great importance in the sense that it shows how DNA vaccine becomes very effective via topical application if the keratinocytic layer is removed. Other methods can be used to remove the layer, such as multiple application of adhesive tape in combination with a low concentration of SDS and/or urea cream, or a gene gun. Human skin has 7–10 keratinocytic layers on the epidermis, whereas mouse skin has only two to four layers. It would be more difficult for DNA plasmids to be taken up by dendritic cells or Langerhans cells without stripping off several layers in humans

ADJUVANT EFFECT

An adjuvant is one of the most important elements in developing an effective HIV vaccine. The use of liposomes may be helpful in this regard, because they are reportedly effective for enhancing immunization (Yasuda *et al*, 1977) and for introducing substances into cells (Flegner *et al*, 1996). Positively charged cationic liposomes were also shown to introduce genes into cells by forming complexes with DNA (Rose *et al*, 1991). A detailed method for cationic liposome preparation has been reported previously (Ishii *et al*, 1997b). As shown in **Fig 2**, the inoculation of DNA vaccine with liposomes induced a substantial DTH reaction. Compared with DNA vaccine plus cationic liposome, topical administration of only the DNA vaccine resulted in weaker DTH response. DNA vaccine plus cationic liposome also induced higher levels of antibody production than DNA vaccine alone. The HIV-1-specific CTL activity was stronger when the DNA vaccine and cationic liposome combination was used. These results showed that cationic liposomes can be highly effective when used with DNA vaccines. Therefore, DNA vaccination with liposomes can induce strong humoral and cell-mediated immune responses.

EFFECT OF IMMUNOMODULATORY MOLECULES: IL-12 AND GM-CSF

To examine the effects of cytokine-expression plasmid on DNA vaccine, pIL-12 and/or pGM-CSF were coimmunized with DNA vaccine (Liu *et al*, in press). When DNA vaccine was applied in combination with pIL-12 and/or pGM-CSF, DTH was significantly enhanced as compared with application of DNA vaccine alone (**Fig 3**). Coadministration of DNA vaccine with pIL-12 and/or pGM-CSF induced a stronger CTL response and higher titer of HIV-1-specific serum IgG and IgA antibodies, when compared with DNA vaccine alone. These results demonstrated that improved topical application of HIV-1 DNA vaccine with pIL-12 and pGM-CSF could induce a significant level both of cell-mediated immune response (DTH and CTL) and of antibody production.

TH1 AND TH2

We studied the profile of cytokines activated with DNA vaccine alone or with pIL-12 and/or pGM-CSF by topical application. As shown in **Fig 4**, a significant increase in IFN-γ and IL-4 production was observed in mice with the use of DNA vaccine. The increase in IL-4 production was greater than that of IFN-γ when topical application rather than i.m. immunization was used. These results suggest that topical application of DNA vaccine preferentially activates Th2 cytokine production in comparison with i.m. immunization.

This study demonstrated that strong HIV-1-specific immune response was induced by topical application. In humoral response, IgG was the most prominently induced. Moreover, this humoral response was enhanced by coimmunization with the plasmid encoding GM-CSF. But in the IgG subclass, the IgG1/IgG2a ratio of topical application was higher than that of i.m. immunization. Cellular immunity, as represented by DTH and CTL, was also enhanced by coapplication of IL-12-expressing plasmid. We detected high levels of IL-4 production and substantial levels of IFN-γ in the culture supernatant of splenocytes with antigens. Taken together, the topical application of DNA vaccine seems to induce both Th2 and Th1, but predominantly Th2 rather than Th1 cytokine response. We need to resolve the problem of variation in Th1–Th2 interaction caused by different immunization routes.

IMPORTANCE OF LANGERHANS CELLS

The effect of anti-CD11c antibody on skin-mediated immune responses was analyzed, and both DTH (footpad swelling) and antibody response (IgG) were assayed. As shown in **Fig 5**, the immune response was significantly inhibited by the injection of anti-CD11c monoclonal antibody or anti-I-A^d/I-E^d monoclonal antibody (Liu *et al*, in press). It suggests that the immune response induced by topical application to the skin is mediated by the I-A/I-E and/or CD11c positive cells, presumably

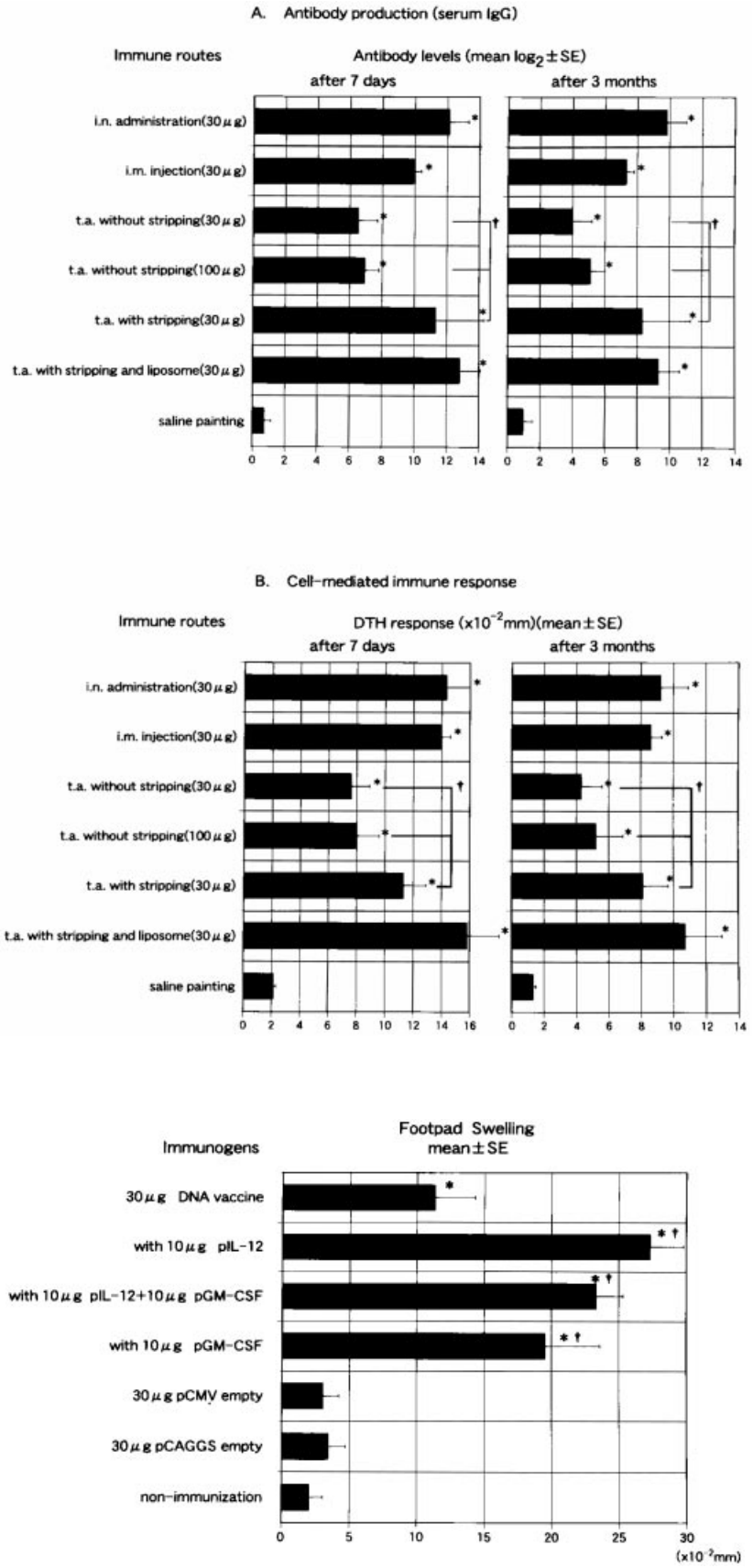

Figure 2. Comparison of immune responses using three routes of immunization. On days 0, 7, and 14, 5–6 BALB/C mice were immunized i.n., i.m., or topically (t.a.) with the indicated volume of IIIB/REV DNA vaccine. Serum IgG and DTH (footpad swelling) response were assayed after 7 d and 3 mo. Similar results were obtained in two other separate experiments. *$p < 0.01$ *versus* nonimmune controls (saline application); †$p < 0.05$ between topical application with stripping and without stripping.

Figure 3. Footpad swelling response in mice induced by topical application of DNA vaccine with or without IL-12, GM-CSF expression plasmids. BALB/C mice were treated three times with 30 μg DNA vaccine alone or with 10 μg pIL-12 and/or pGM-CSF. Seven days after vaccination, the footpad-swelling assay was carried out. The response of each group represents the mean ± SE of 5–6 mice. *$p < 0.05$ *versus* pCMV empty and/or pCAGGS empty groups; †$p < 0.05$ *versus* DNA vaccine only groups. Similar results were obtained in two other separate experiments.

Langerhans cells in the epidermis and dermis. Robinson *et al* (1999) reported that strong immune response induced by intradermal injection of DNA vaccine indicated the importance of Langerhans cells in the dermis. We agree with Robinsons' hypothesis (1999), and understand the need to analyze Langerhans cells in the epidermis and dermis.

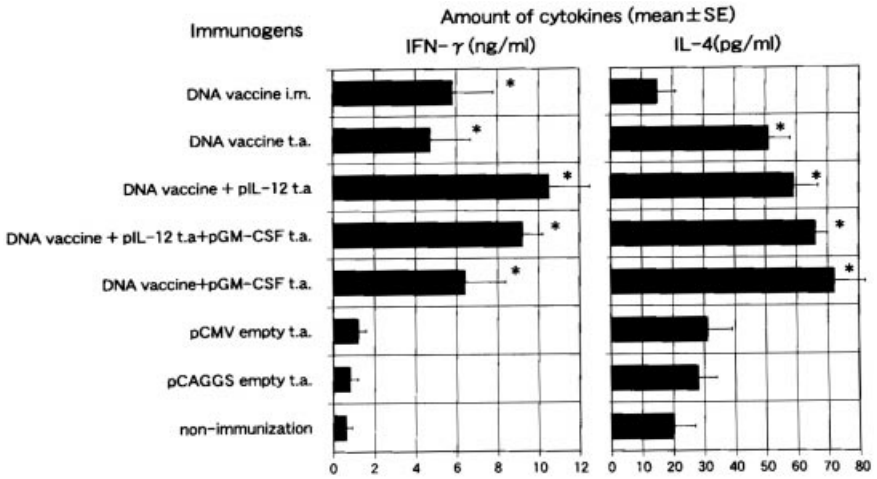

Figure 4. Cytokine profiles in supernatants of lymphoid cell cultures harvested from mice immunized with DNA vaccine with or without cytokine expression plasmids. BALB/C mice were inoculated i.m. once with 10 μg of DNA vaccine or by topical application (t.a.) three times with 30 μg of DNA vaccine alone or combined with pIL-12 and/or pGM-CSF. Seven days after the final immunization, spleen cells were harvested from the mice and cultured with HIV-1 IIIB peptide. Cytokine levels in the culture supernatants were assayed using commercial ELISA kits. The levels of each group represented the mean ± SE obtained in 4–6 mice. The results of two other separate experiments showed similar results. *$p < 0.05$ between nonimmune controls and each immunized group.

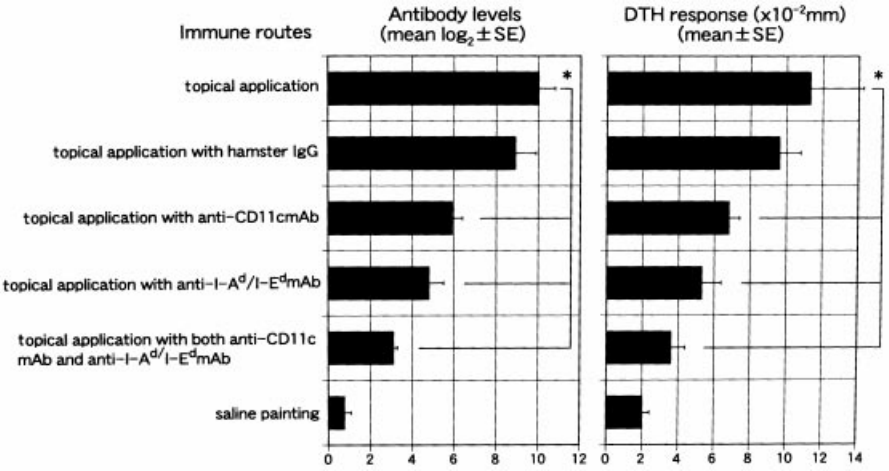

Figure 5. Inhibition of DNA vaccine-mediated immune responses by intradermal injection of anti-CD11c antibody. One hour after application of DNA vaccine (30 μg), mice were given an intradermal injection of 50 μg normal hamster IgG or anti-CD11c Ab from hamsters. These procedures were repeated on days 0, 7, and 14. Seven days after the last immunization, both the DTH and the antibody response IgG were assayed. Data represent mean ± SE of 3–5 mice. *$p < 0.05$ between topical application and that with anti-CD11c monoclonal antibody or/and anti-I-A^d/I-E^d monoclonal antibody.

CONCLUSION

The classical or genetic vaccination methods that use needles or various infectious vector systems suffer from problems of administration expense and patient compliance. In this study we demonstrated that application of DNA vaccine to animal skin induced both cellular and humoral immune response against HIV envelope protein. We previously reported several delivery systems for DNA vaccine (Bukawa *et al*, 1995; Okuda *et al*, 1995; Okada *et al*, 1997; Toda *et al*, 1997; Tsuji *et al*, 1997a, b; Sasaki *et al*, 1998), including i.m. and i.n. administration to induce immune response against HIV protein. Immunization including subcutaneous or intradermal immunization has been widely studied (Hengge *et al*, 1996; Sato *et al*, 1996; Tan *et al*, 1996; Tutin *et al*, 1999). Topical application tends to be more potent in inducing both humoral and cellular immune response, and it requires no invasive maneuvers such as needle injection in developing countries. Therefore, this route is potentially useful for immunization against HIV. We also applied our DNA vaccine without removing hair or keratinocytes (Fan *et al*, 1999), but these methods did not work. We need to try some more convenient topical application system. The gene gun propels plasmids into cells near the surface of the body, typically those of the skin or mucous membranes (Weiner and Kennedy, 1999). In addition, adjuvant-like cationic liposomes, or immunomodulatory molecules such as GM-CSF and IL-12 expression plasmids, were proven to greatly modify the immune response by topical coadministration. These effects of cytokine expressing plasmids were able to efficiently improve the vaccination effect.

This work was partially supported by a Grant-in-Aid for Scientific Research (C) (11670844) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

Bukawa H, Sekigawa K, Hamajima K, Fukushima J, Yamada Y, Kiyono H, Okuda K: Neutralization of HIV-1 by secretory IgA induced by oral immunization with a new macromolecular multicomponent peptide vaccine candidate. *Nature Med* 1:681–685, 1995

Disis ML, Bernhard H, Shiota FM, *et al:* Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccine. *Blood* 88:202–210, 1996

Donnelly JJ, Ulmer JB, Shiver JW, Liu M: DNA vaccines. *Ann Rev Immunol* 15:617–648, 1997

Fan H, Lin Q, Morrissey GR, Khavari PA: Immunization via hair follicles by topical application of naked DNA to normal skin. *Nature Biol* 17:870–872, 1999

Flegner PL, Tsai YJ, Mashall J, Cheng SH, Sukhu L, Wheeler CJ, Manthorpe M: Improved cationic lipid formulations for in vivo gene therapy. *Ann NY Acad Sci* 772:126–139, 1996

Hassett DE, Whitton JL: DNA immunization. *Trend Microbiol* 4:307–312, 1996

Hengge UR, Walker PS, Vogel JC: Expression of naked DNA in human, pig and mouse skin. *J Clin Invest* 97:2911–2916, 1996

Ishii N, Fukushima J, Kaneko T, *et al:* Cationic liposomes are a strong adjuvant for a DNA vaccine of human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses* 13:1421–1428, 1997b

Ishii N, Sugita Y, Nakajima H, *et al:* Genetic control of immune responses to HIV-1 *env* DNA vaccine. *Microbiol Immunol* 41:421–425, 1997a

Liu L-J, Watabe S, Yang J, *et al:* Topical application of HIV DNA vaccine with cytokine-expression plasmids induces strong antigen-specific immune responses. *Vaccine*, in press

Okada E, Sasaki S, Ishii N, *et al:* Intranasal immunization of a DNA vaccine with IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. *J Immunol* 159:3638–3647, 1997

Okuda K, Bukawa H, Hamajima K, *et al:* Induction of potent humoral and cell-mediated immune responses following direct injection of DNA encoding the HIV type 1 env and rev gene products. *AIDS Res Hum Retroviruses* 11:933–943, 1995

Robinson HL, Montefiori DC, Johnson RP, *et al:* Neutralizing antibody-independent containment of immunodeficiency virus challengers by DNA priming and recombinant pox virus booster immunizations. *Nature Med* 5:526–534, 1999

Rose JK, Buonocore L, Whitt M: A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *Biotechniques* 10:520–525, 1991

Sasaki S, Sumino K, Hamajima K, *et al:* Induction of systemic and mucosal immune responses to human immunodeficiency virus type-1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes. *J Virol* 72:4931–4939, 1998

Sato Y, Roman M, Tighe H, *et al:* Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 273:352–354, 1996

Tan J, Newton CA, Djeu JY, *et al:* Injection of complementary DNA encoding interleukin-12 inhibits tumor establishment at a distant site in a murine renal carcinoma model. *Cancer Res* 56:3399–3403, 1996

Toda S, Ishii N, Kusakabe K, *et al:* HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon-γ antibody. *Immunol* 92:111–117, 1997

Tsuji T, Hamajima K, Fukushima J, *et al:* Enhancement of cell-mediated immunity against HIV-1 induced by coinoculation of plasmid-encoded HIV-1 antigen with plasmid expressing IL-12. *J Immunol* 158:4008–4014, 1997a

Tsuji T, Hamajima K, Ishii N, *et al:* Immunodulatory effects of a plasmid expressing B7–2 on human immunodeficiency virus-1-specific cell-mediated immunity induced by a plasmid encoding the viral antigen. *Eur J Immunol* 27:782–787, 1997b

Tutin T, Storkus WJ, Falo LD Jr: DNA immunization targeting the skin: molecular control of adaptive immunity. *J Invest Dermatol* 111:183–188, 1999

Weiner DR, Kennedy RC: Genetic vaccine. *Sci Am* July:34–41, 1999

Xin K-Q, Hamajima K, Sasaki S, *et al:* Intranasal administration of HIV-1 DNA vaccine with interleukin-2 expression plasmid enhance cell-mediated immunity against HIV-1. *Immunology* 94:438–444, 1998

Yasuda T, Dancey GF, Kinsky SC: Immunogenic properties of liposomal model membranes in mice. *J Immunol* 119:1863–1867, 1977